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Discrimination of fresh frozen non-tumour and tumour brain tissue using spectrochemical analyses and a classification model

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Abstract

Introduction: In order for brain tumours to be successfully treated, maximal resection is beneficial. A method to detect infiltrative tumour edges intraoperatively, improving on current methods would be clinically useful. Vibrational spectroscopy offers the potential to provide a handheld, reagent-free method for tumour detection.
Purpose: This study was designed to determine the ability of both Raman and Fourier-transform infrared (FTIR) spectroscopy towards differentiating between normal brain tissue, glioma or meningioma.
Method: Unfixed brain tissue, which had previously only been frozen, comprising normal, glioma or meningioma tissue was placed onto calcium fluoride slides for analysis using Raman and attenuated total reflection (ATR)-FTIR spectroscopy. Matched haematoxylin and eosin slides were used to confirm tumour areas. Analyses were then conducted to generate a classification model.
Results: This study demonstrates the ability of both Raman and ATR-FTIR spectroscopy to discriminate tumour from non-tumour fresh frozen brain tissue with 94% and 97.2% of cases correctly classified, with sensitivities of 98.8% and 100% respectively. This decreases when spectroscopy is used to determine tumour type.
Conclusion: The study demonstrates the ability of both Raman and ATR-FTIR spectroscopy to detect tumour tissue from non-tumour brain tissue with a high degree of accuracy. This demonstrates the ability of spectroscopy when targeted for a cancer diagnosis. However, further improvement would be required for a classification model to determine tumour type using this technology, in order to make this tool clinically viable.

Key Words: Brain tumours, Classification model, Intraoperative diagnosis, Neurosurgery, Spectrochemical analyses

Introduction

Brain tumours account for 3% of all tumours diagnosed annually, with incidence rates increasing approximately 15% over the last decade[1]. Whilst they comprise a small proportion of all cancers diagnosed per year, the difficulty of complete removal of the tumour is inherent. High-grade tumours can be infiltrative with up to 75% of tumour resections thought to leave behind viable tumour [2]. Current techniques include the use of 5-aminolevulinic acid (5-ALA), a fluorescent compound to fluoresce tumour cells to enable the surgeon to visualise them more easily. This allows real-time feedback and does not rely on repeat intraoperative imaging [3,4]. A tool that could either improve, or work in conjunction with 5-ALA could prove useful to neurosurgeons, improving resection rates further and thus, hopefully, disease-free survival.

Vibrational spectroscopic techniques have been in use for many years, including two forms: Raman and attenuated total reflection Fourier-transform infrared (ATR-FTIR) spectroscopy. These are complimentary techniques producing results based upon vibrations within the chemical bonds of the interrogated sample. Studies on many tumour types comprising both tissue and biofluids, have shown promising results, and use a combination of both ATR-FTIR and Raman spectroscopy, with an ability to detect tumour from non-tumour cases [5-18]. Both Hands *et. al.* and our group have demonstrated separation of patients with and without primary brain tumours and metastatic lesion using blood serum and plasma with high sensitivities and specificities [19-21].

Much of the current tissue-based work has been performed on formalin-fixed paraffin-embedded (FFPE) tissue. This presents a challenge, as even with dewaxing, the paraffin present may still interfere with spectra and hence results [22,23]. It has also

been shown to vary depending on the substrate upon which the tissue is placed [24]. Previous studies have demonstrated the ability to differentiate low-grade and high-grade gliomas, meningiomas, and metastatic tumours, as well as producing a database of paediatric tumours to produce a classification model[25,26]. Differentiation of metastatic brain tumours has proven more challenging as results found some tumour types overlapped, for example adenocarcinomas, though there were points of differentiation identified in tumours of differing phenotype [27,28]. New studies from Desroches *et. al.* have demonstrated the use of a hand held Raman probe intraoperatively obtaining an accuracies of 84-87% when differentiating tumour from non tumour brain tissue [29,30].

No studies have yet been done using fresh frozen tissue to differentiate different types of adult primary brain tumours. Whilst frozen tissue also has its own complications, the closer tissue is to its natural fresh state, the more relatable the results become [22]. This novel study therefore aims to determine the ability of both ATR-FTIR and Raman spectroscopy to classify non-tumour brain from gliomas and meningiomas using fresh frozen tissue in order to reduce the signal received from tissue fixation. To the authors' knowledge this is the first study performed using fresh frozen tissue, comparing both Raman and ATR-FTIR spectroscopy on adult brain tumours. If successful, results from this study have the potential to open the door towards intraoperative use of spectroscopy to delineate tumour from non-tumour brain tissue. This would provide a major advance in the intraoperative diagnosis of brain tumours.

Methods

Ninety-six cases of fresh frozen brain tissue comprising primary brain tumours both gliomas of varying grades and meningiomas, along with normal brain were selected from the Brain Tumour North West tissue bank, with ethical approval (NRES14/EE/1270). This tissue has been retrieved from the patient and then snap frozen on arrival within the histopathology department. Frozen sections are cut and allowed to defrost prior to spectral acquisition. This tissue was chosen for analysis as it has not previously been formalin-fixed and therefore is closest to fresh tissue allowable given the number of cases tested. The cases used in the study are shown in Table 1 below, categorised by tumour type.

Ten-µm-thick frozen sections were placed onto 25 × 25 × 1 mm Raman-grade calcium fluoride (CaF_2)-coated slides (Cyrstan Limited). A matched 4-µm-thick section stained with haematoxylin and eosin (H&E) was then cut to allow viable tumour areas to be marked and confirmed. Following this, spectrochemical measurements were performed on the unstained samples using both Raman and ATR-FTIR spectroscopy, focussed on the viable tumour areas.

Raman spectroscopy

Spectra were taken from 20-25 sampling points within the tumour tissue area using a Horiba Jobin-Yvon LabRAM HR800 spectrometer over 1800-400 cm^{-1} wavenumbers. An air-cooled CLDS point mode diode 785 nm laser with a single edge filter (cut off to 100 cm^{-1}) and an output power of 300 mW. This was done with a confocal hole of 100 µm at a grating of 300 gr/mm and a ×50 objective. For each spectrum, 2 accumulations each over 30 seconds were acquired.

ATR-FTIR Spectroscopy

The ATR-FTIR spectroscopy measurements were performed on an Agilent Cary-600 Series FTIR spectrometer. Measurements were taken in transmission mode with 32 co-added scans over a range of 4000-400 cm^{-1} and a resolution of 4 cm^{-1}. A background scan was taken prior to each sample with the same settings. Twenty sampling points were selected within each viable tumour area.

Computational analysis

Data collection and manipulation was performed within a MATLAB R2014b environment (MathWorks Inc., USA) using PLS Toolbox 7.9.3 (Eigenvector Research Inc., USA) with specimens first assigned to training, validation and test groups using the Kennard-Stone algorithm (see Table 2), where 70% of samples were placed into training and 15% each into validation and test groups.

Pre-processing using Savitzky-Golay smoothing followed by multiplicative scatter correction (MSC), baseline correction, and vector normalization were performed. The spectra were cut from 1800-500 cm^{-1} [see Supplementary Information (SI) Figures S1 and S5)]. Following on from this, principal component analysis with linear discriminant analysis (PCA-LDA) or quadratic discriminant analysis (PCA-QDA), and genetic algorithm with LDA (GA-LDA) or quadratic discriminant analysis (GA-QDA) were performed in order to determine the best analytical method [31]. The training samples were used for model construction and the test set for the final classification evaluation. The validation group was used to prevent overfitting, once the model parameters are optimized according to the classification performance of this data set, making it sure that the training fitting performance is in accordance with the validation response. The optimum number of variables for GA-LDA/QDA was performed based on the average risk G of misclassification in the validation set [32]. The

GA routine was carried out during 40 generations with 80 chromosomes each. Crossover and mutation probabilities were set to 60% and 10%, respectively. Moreover, the algorithm was repeated three times, starting from different random initial populations. The best solution (in terms of the fitness value) was employed. LDA and QDA were employed to the PCA scores and GA selected variables based on a Mahalanobis distance calculation between the classes [32].

Results

Raman Spectroscopy

From the 96 cases, 1911 spectra were collected. During pre-processing 30 spectra were removed due to poor quality, observed by a Hotelling T^2 *versus* Q residuals test. As in Table 2, tumours were classified by type rather than grade. Following pre-processing there were 159 spectra in class 1, 666 in class 2 and 1056 in class 3. Firstly, comparison was done between normal and tumour tissue, grouping both meningiomas and gliomas together (Figure 1, Table 3). This demonstrates that 94% of the cases were correctly classified as either tumour or non-tumour brain tissue, with a sensitivity of 98.8% and specificity of 41.7%.

Following on from this the model was tested to determine if it could identify normal from meningioma from glioma (Figure S2 and Table 4). When asked to determine tumour by type the overall classification accuracy fell to 63.1%. Normal brain tissue was still detected with an accuracy of over 90%. Comparisons between each individual group are shown in the supplementary material.

ATR-FTIR Spectroscopy

The process was then repeated for ATR-FTIR spectroscopy. From the 96 cases, 1919 spectra were collected; again during pre-processing 38 spectra were removed due to

poor quality, observed by a Hotelling T^2 *versus* Q residuals test. Spectra were divided as above. Following pre-processing there were 159 spectra in class 1, 666 in class 2 and 1056 in class 3. As for the Raman spectra, firstly, normal was compared to tumour (meningioma and glioma) with GA-QDA providing the best results, with a classification accuracy of 97.2% (Figure S7, Table 5). The sensitivity was 100% and specificity 66.7%.

When comparing if the classification model could correctly identify normal versus meningioma versus glioma the accuracy fell to 79.2%, (Figure 2 and Table 6) however was still above that achieved with the Raman spectroscopy (63.1%). FTIR also gave higher accuracy results when comparing tumour to no tumour, 97.7% compared to 94%.

Discussion

The ability of vibrational spectroscopic techniques to detect brain tumours with both blood components [5-8,19-21] and formalin-fixed tissue [25,28] has been previously demonstrated with high accuracy levels. Studies using fresh frozen brain tissue are few and far between, with one study within the paediatric field showing an ability to detect different tumour types and a second trialling a hand held Raman machine intraoperatively slowly moving forward [26, 29,30]. This study aimed to compare both Raman and ATR-FTIR spectroscopy using fresh tissue, which had previously only been frozen, in order to determine which provided the most accurate classification results as a precursor to developing a tool for intraoperative detection of primary brain tumours. We have shown that as compared to normal brain tissue, ATR-FTIR and Raman spectroscopy can both detect normal from tumour tissue with a high degree of accuracy (97.7% and 94%, respectively). However, when asked to determine tumour

type, the accuracy of both techniques drops (79.2 and 63.1%, respectively). FTIR spectroscopy was however, considerably higher than Raman, perhaps demonstrating it is better placed to differentiate between the tumour types. The accuracy does though remain greatly below that offered by a conventional intraoperative smear diagnosis and thus would require improvement in order to be a clinically diagnostic tool. Though, these rates would allow for intraoperative delineation of tumour versus normal. Importantly, the sensitivity when comparing normal to tumour is high (87.1-100%), meaning we are not over diagnosing tumours. The specificities are lower, though in this situation where a surgeon is aware of the presence of a tumour, high sensitivity remains the priority. One limitation of the study is the low number of 'normal' *i.e.* non-tumour cases tested (*n=8*) as the majority of patients undergoing neurosurgery have a tumour. This is due to the low number of normal fresh frozen cases available within the brain bank. Therefore, if used clinically, a much larger number of normal samples is needed, the ability to test more background non-tumour brain is likely to improve the classification accuracy and specificity.

Moving forward, discussion with clinicians is also required to determine what is needed from an intraoperative diagnostic tool; *i.e.*, cancer *versus* non-cancer or a defined tumour type and grade. The output from the machine for the surgeon then also needs to be defined. The use of a sound has previously been proposed, to allow the surgeon real time feedback of the spectroscopic output [33]. Easy to interpret, quick results would be required. Inter-user and inter-site consistency is also required.[34]

Overall, we have shown in this study and other research[35] that spectroscopy may have potential in the diagnosis of intraoperative brain tumours; however, further work to improve classification would be required prior to clinical implementation.

Further work to allow for comparison of primary to metastatic tumours would also prove useful in providing clinical useful information in real time.

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Table 1 Tumour samples selected for analysis, broken down by tumour type and WHO grade.

	N	WHO Grade 1	WHO Grade 2	WHO Grade 3	WHO Grade 4	No Grade
All Cases	96	25	11	14	33	5
Normal brain	8	N/A	N/A	N/A	N/A	N/A
Gliomas	54	1	6	11	33	3
Meningiomas	34	24	5	3	N/A	2

Table 2 Number of samples within the training, validation and test groups based on the application of the Kennard-Stone algorithm.

Class	Training	Validation	Test
Normal	111	24	24
Meningioma	466	100	100
Glioma	739	158	159

Table 3 Results for classification models for normal *versus* tumour (meningioma and glioma) using Raman spectroscopy. Highlighted in red is the best classification model.

	PCA-LDA	PCA-QDA	GA-LDA	GA-QDA
Accuracy (%)	93.3	94.0	91.5	91.8
Sensitivity (%)	98.4	98.8	97.3	97.7
Specificity (%)	37.5	41.7	29.2	29.2
PPV (%)	94.4	94.8	93.7	93.7
NPV (%)	69.2	76.9	50.0	53.8
Youden's Index	35.9	40.5	26.5	26.8

Correct Classification (%)	Training	Validation	Test
PCA-LDA	85.5	91.4	93.3
PCA-QDA	93.4	94.3	94.0
GA-LDA	84.3	90.7	91.5
GA-QDA	86.6	91.8	91.8

Table 4 Results for classification models of normal *versus* meningioma *versus* glioma using Raman spectroscopy.

	Normal		Meningioma		Glioma	
	PCA-LDA	GA-LDA	PCA-LDA	GA-LDA	PCA-LDA	GA-LDA
Accuracy (%)	92.9	92.6	69.5	68.4	63.6	62.4
Sensitivity (%)	33.3	29.2	33.7	36.6	86.6	82.8
Specificity (%)	98.4	98.4	89.5	86.2	35.2	36.8
PPV (%)	66.7	63.6	64.2	59.7	62.7	62.6
NPV (%)	94.1	93.7	70.7	70.9	67.7	63.0
Youden's Index	31.8	27.6	23.2	22.8	21.8	19.6

Correct Classification (%)	Training	Validation	Test
PCA-LDA	59.0	62.5	63.1
GA-LDA	66.1	68.9	61.7

Table 5 Results of classification models for normal *versus* tumour (meningioma and glioma) using IR spectroscopy, with the best classification model highlighted in red.

	PCA-LDA	PCA-QDA	GA-LDA	GA-QDA
Accuracy (%)	92.1	87.1	94.1	97.7
Sensitivity (%)	98.1	87.1	100	100
Specificity (%)	12.5	87.5	16.7	66.7
PPV (%)	93.7	98.9	94.1	97.5
NPV (%)	33.3	33.9	100	100
Youden's Index	10.6	74.6	16.7	66.7

Correct Classification (%)	Training	Validation	Test
PCA-LDA	81.1	92.9	90.5
PCA-QDA	93.3	86.2	84.5
GA-LDA	91.5	95.4	92.9
GA-QDA	96.7	97.9	97.2

Table 6 Results of the classifcation models for normal *versus* meningioma *versus* glioma using IR spectroscopy.

	Normal		Meningioma		Glioma	
	PCA-LDA	GA-LDA	PCA-LDA	GA-LDA	PCA-LDA	GA-LDA
Accuracy (%)	90.8	95.8	73.1	83.4	64.0	79.2
Sensitivity (%)	8.3	50.0	26.0	56.0	96.2	98.1
Specificity (%)	98.5	100	98.9	98.4	22.6	54.8
PPV (%)	33.3	100	92.9	94.9	61.4	73.6
NPV (%)	92.1	95.6	71.0	80.4	82.4	95.8
Youden's Index	6.8	50.0	24.9	54.4	18.8	53.0

Correct Classification (%)	Training	Validation	Test
PCA-LDA	62.8	61.7	64.0
GA-LDA	83.1	84.0	79.2

Figure Captions

Figure 1 Graphical representations of normal *versus* tumour (Meningioma and glioma) using Raman spectroscopy. (A) PCA-LDA, (B) PCA-QDA, (C) GA-LDA, (D) GA-QDA.

Figure 2 Graphical representations of normal *versus* tumour (meningioma and glioma) using IR spectroscopy. (A) PCA-LDA, (B) PCA-QDA, (C) GA-LDA, (D) GA-QDA.